

IMMUNOHISTOCHEMICAL ANALYSIS OF XRCC5, MDH2 AND ACO2 IN PROSTATE CANCER

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LASSILA ARTTURI: IMMUNOHISTOCHEMICAL ANALYSIS OF XRCC5, MDH2 AND ACO2 IN PROSTATE CANCER

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Prostate cancer is a very heterogeneous disease and its prognostic markers and oncogenes are under intensive research. Goal would be to assess patient's unique "mutational fingerprint" and determine the best possible treatment for each individual cancer.

This study examined the possible connection between prostate cancer and three proteins, mitochondrial aconitase (*ACO2*), malate dehydrogenase (*MDH2*) and nuclear Ku80 (*XRCC5*). These were noticed to have an altered expression levels in prostate cancer in mass spectrometry analyses performed earlier.

Study material consisted of previously obtained TMA-specimens with 502 primary prostatectomy (PP) and 148 hormone-refractory (HR) samples. These were stained immunohistochemically and observed with a virtual microscope. Possible protein expression was graded according to the staining intensity with a scale from 0 to 3.

Of these proteins, *ACO2* and *MDH2* were noticed to have a statistically significant (p-value < 0.05) association with a proliferation marker Ki-67 which is expressed in actively dividing cells, including cancerous cells. This might suggest that these proteins are overexpressed in prostate cancer but more studies with larger sample sizes will be needed to verify the results.

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1 INTRODUCTION

References for this work were searched through Medline and PubMed -databases and with Google Scholar -search engine. References are original and review articles and websites, such as NCBI (National Center for Biotechnology Information). A textbook was also used for reference.

1.1 Normal physiology of the prostate

Robbins & Cotran Pathologic Basis of Disease, 9th edition (1) was used as a reference for chapters 1.1 and 1.2. Prostate is an organ situated retroperitoneally, encircling the neck of the bladder and urethra with the ejaculatory duct going through it and connecting with urethra inside the gland. Prostate can be divided anatomically and biologically into four distinct zones: periurethral, central, transitional and peripheral zone. Different pathologies arise often in distinct areas. For example, most benign hyperplasias develop in the transitional zone where they can obstruct the urethra and cause urinary retention and pain. On the other hand, carcinomas arise most often in the peripheral zone where they can be palpable during digital rectal examination.

Histologically prostate contains glands, fibromuscular stroma between these glands and neuroendocrine cells. Glands are composed of two epithelial cell layers: cuboidal basal cells and columnar secretory cells. They are often large with papillary infoldings. Fibromuscular stroma has smooth muscle cells, fibroblasts and other cell types typical of glandular stroma.

The normal function of the prostate is to secrete different enzymes, proteins and nutrients to the seminal fluid as it passes through the prostate during ejaculation. These different

molecules function, among other things, to liquefy the sperm and provide nutrients for the sperm cells.

1.2 Pathogenesis of prostate cancer

Great majority of cancers arising from the prostate are multifocal, gland-derived adenocarcinomas. Histologically carcinoma glands are only one cell layer thick with basal cell layer missing. Glands are often smaller than their benign counterparts and more circular. The nucleus in luminal cells is often large with one or several prominent nucleoli visible with normal light microscope. Mitotic figures are rare and general cell pleomorphisms aren't that common.

1.2.1 Mutational heterogeneity

The course of prostate cancer varies greatly among individuals. To some, cancerous mutations never manifest as clinical symptoms and yet to others, cancer grows and spreads quickly sending metastases to other organs, particularly to bones in the case of prostate cancer. The role of genetic and epigenetic variations between different cancer genomes has been under intensive study as researchers have been trying to find correlations between mutations and clinical courses. A lot has been accomplished with a molecular classification of carcinomas according to the specific genetic aberrations. (2).

There are a couple of validated germline mutations contributing to the prostate cancer risk. Only one is a definite prostate cancer predisposition gene, *HOXB13*. The other is *BRCA2*, which is associated with breast and ovarian cancer too. (2).

The most common somatic mutation specific to prostate cancer is a fusion of an androgen-regulated promotor area with an E26 transformation-specific (ETS) family transcription factor. The most common form is *TMPRSS2-ERG* fusion. It is found in between 40-50 %

of tumor foci. Despite its large prevalence, its effect to tumor prognosis is uncertain. Nearly 90 % of ETS-fusions are of *ERG*-type. (3). Other common possibilities are *ETV1*, *ETV4*, or *FLI1* (4).

DNA-copy number alterations are also common in prostate cancer. Somatic point mutations on the other hand aren't that prevalent (5). The genes mutated most often in prostate cancer are *SPOP*, *TP53*-deletion, *FOXA1*-amplification and *PTEN*-deletion. M. Loda et al. (4) found that 74% of 333 prostate cancer tumors could be subdivided to seven roughly distinct molecular classes: fusions involving (1) *ERG*, (2) *ETV1*, (3) *ETV4* or (4) *FLI1* or mutations in (5) *SPOP*, (6) *FOXA1* or (7) *IDH1*. Especially *SPOP* mutations and chromosomal fusions have been found to be mutually exclusive. (4,5).

1.2.2 The Role of androgens

Androgens, especially dihydrotestosterone (DHT) have long been known to control critical cell survival and growth in prostate tissue. Type 2 5 α -reductase, mostly in stromal cells, converts testosterone from the blood to DHT which binds to nuclear androgen receptor (AR) in the glandular cells and enables the transcription of androgen-dependent genes. (1).

In prostate cancer, the dependence on androgens persists. Standard of care for locally advanced or metastasised carcinoma has long been chemically or surgically induced castration where circulating testosterone levels drop dramatically. Tumor often shrinks and stops advancing, but in most cases, it resurfaces with an acquired resistance to this androgen deprivation therapy (ADT). This malignancy is called a castration-resistant prostate cancer (CRPC) or hormone-refractory (HR) cancer. (6).

Common mutations leading to castration-resistance include AR amplification as well as mutations in proteins that physically interact with the AR, such as *FOXA1*, *MLL2*, *UTX* and *ASXL1*. These proteins function as epigenetic modifiers and co-factors, for example *MLL2* encodes a histone methyltransferase which affects the expression of androgen receptor. (7).

1.3 Diagnosis and biomarkers in prostate cancer

Essential part of the prognosis and treatment of prostate cancer is the early discovery of the disease. Biomarkers play a key role here as they are the means to acquire information from the tissues with minimal intervention. PSA has been the golden standard for diagnosing prostate cancer for over 25 years. It is however, not without its flaws; PSA levels rise in many common non-cancerous situations like inflammation and benign prostate hyperplasia. This leads to over-diagnosing and unnecessary prostate biopsies and because of these, psychological stress, loss of bodily function and pain ensues. (3). Thus, it is important to find more potent biomarkers with better sensitivity and specificity.

Many promising candidates have emerged like urinary tests for *TMPRSS2-ERG* fusion and *PCA3*-overexpression. Other interesting possibilities are for example testing for circulating tumor cells and exosomes from the blood. These new methods are still used in conjunction with PSA but they provide enhanced positive and negative predictive values compared to PSA-test alone. (8).

1.4 The epidemiology of prostate cancer

Prostate cancer is the most common cancer diagnosed with men in developed countries with over 750 000 new cases annually. Worldwide there are estimated to be more than a million new cases and over 300 000 deaths every year as of 2012. (9). Statistics conducted in the United States show that prostate cancer accounts for 29% of all cancers diagnosed with men there (10).

Age, ethnic background and family history are the most important risk factors in prostate cancer. People with African descent have the highest incidence, Asian men the most infrequent and Caucasians are in between. Cancer of the prostate, like majority of cancers, is typically a disease of older individuals, over 60 years most often. Positive

family history of the disease increases the risk for first degree relatives 2 to 4 times higher than that of the general population. (1,2,4).

1.5 XRCC5, MDH2 and ACO2 in prostate cancer

Mass spectrometry studies performed earlier showed that two mitochondrial proteins, aconitase (*ACO2*) and malate dehydrogenase (*MDH2*), and one nuclear DNA-repair protein, Ku80 (*XRCC5*) were having an altered expression levels in prostate cancer. This follow-up study aims to verify these expression level deviations using primary prostatectomy and castration resistant prostate cancer samples.

1.5.1 ACO2

Aconitase has a crucial part in a cell's energy metabolism. It catalyses the conversion from citrate to iso-citrate, the second reaction in the kreb's cycle. (11,12). In prostate tissue, this enzyme is normally inhibited causing accumulation of citrate. This excess citrate is secreted into the prostatic fluid where it serves as a nutrient for the semen. (13,14).

Zinc has a pivotal role in inhibiting m-aconitase activity. Prostate tissue accumulates the greatest amount of zinc in the whole body, a part of which is stored in cells and a part secreted into the prostatic fluid. (15). In prostate cancer, this zinc accumulation system does not work. The protein supposed to transfer zinc across the cell membrane (ZIP1) is downregulated and intracellular zinc levels drop as much as 70-80 %. Thus, m-aconitase can convert citrate to iso-citrate, continuing kreb's cycle towards oxidative phosphorylation. This change provides much needed energy for the growing and dividing cancer cells. (13,15).

One study found that there was no significant change in the amount of m-aconitase in cancer cells and concluded that the increase in m-aconitase activity is due to the ceasing

of inhibition by zinc (16). Expression levels of metabolic enzymes are often only slightly different in malignant tissues compared to normal cells so the difference is difficult to notice (14).

1.5.2 MDH2

Malate dehydrogenase catalyses the reversible reaction between malate and oxaloacetate in the mitochondria. The enzyme has a pivotal role in attending and controlling for example kreb's cycle, amino acid synthesis and gluconeogenesis. It is controlled allosterically by citrate. (17,18).

Recent studies have found *MDH2* to be over-expressed in uterine cancer as well as in prostate cancer. Suggestions as to how malate dehydrogenase acts in cancer have not yet been conclusive but over-expression is thought to improve on the energy status of the cell and thus provide fuel for mitosis and for example to remove cytostatics from the cancer cell. (19,20). *MDH2* and *ACO2* have both been found to affect the rate of mitosis (21).

1.5.3 XRCC5

Ku80 is in fact part of a heterodimer protein with a Ku70 counterpart. Together they unite with the DNA-PK_{cs} (DNA protein kinase catalytic subunit) to form DNA-PK. Ku is an important factor in the non-homologous end joining (NHEJ) which repairs one of the most harmful damages to DNA, double-strand breaks (DSB). Ku finds the splitted DNA-strands and attaches to them making it possible for other enzymes to join in and begin repairing the DNA. (22,23). Inability to fix DSBs leads to genetic instability which in turn can cause apoptosis or oncogenic transformations (24).

In addition to being an essential factor in NHEJ, Ku also functions as a transcriptional controller. Ku is part of transcription recycling and transcription factor recycling and also a

transcription factor for PSA promoter region. (25). Ku is traditionally expressed in the nucleus but it has also been found on cell membranes on certain cancer cell lines where it takes part in cell adhesion, migration and invasion (26).

2 MATERIAL AND METHODS

The study material consisted of previously obtained primary prostatectomy (PP) and hormone-refractory (HR) prostate tumor samples that had been processed to paraffin blocks using tissue microarray (TMA) technology. These blocks have then been sectioned into thin slices with a microtome and inserted on to individual microscope slides (27). The use of TMAs has been approved by the National Authority for Medicolegal Affairs.

2.1 Immunohistochemical staining

For each protein studied, the right dilution for the primary antibody and the proper buffer solution had to be tested before any final stains. All slides were eventually buffered in the Tris-EDTA buffer with pH 9. Dilutions for the primary antibodies were defined to be 1:500 for *ACO2*, 1:300 for *MDH2* and 1:8000 for *XRCC5*.

The slides were stained automatically using Labvision's Autostainer 480. N-Histofine® Simple Stain MAX PO (multi) detection reagent (anti-mouse and anti-rabbit) was used as a secondary antibody and a horseradish peroxidase (HRP) source. ImmPACT DAB Peroxidase (HRP) Substrate was used as a substrate for the HRP and it provided the dark brown stain used for scoring the possible expression intensity. Hydrogen peroxide (H₂O₂)

was used to minimize the background staining caused by endogenous peroxidase. Hematoxylin was used as a counterstain to visualize the nuclear morphology.

2.2 Analyses with microscope

After the staining protocol was finished, the slides were dehydrated with an increasing ethanol concentration and cleared with xylene. Coverslips were attached to the slides so that they could be more easily handled.

The slides were scanned to the computer with Zeiss Axioskop40 microscope (Carl Zeiss MicroImaging, NY, USA) with 20x objective and a CCD colour camera (QICAM Fast; QImaging, Canada) and a motorized specimen stage (Märzhäuser Wetzlar GmbH, Germany). The slides were digitally examined using IIPZoomViewer- and JVSView- programs. The intensity of the staining was graded from 0 to 3 with zero being no expression at all and 3 with the strongest staining. Figure 1 presents the staining differences using ACO2 expression as an example.

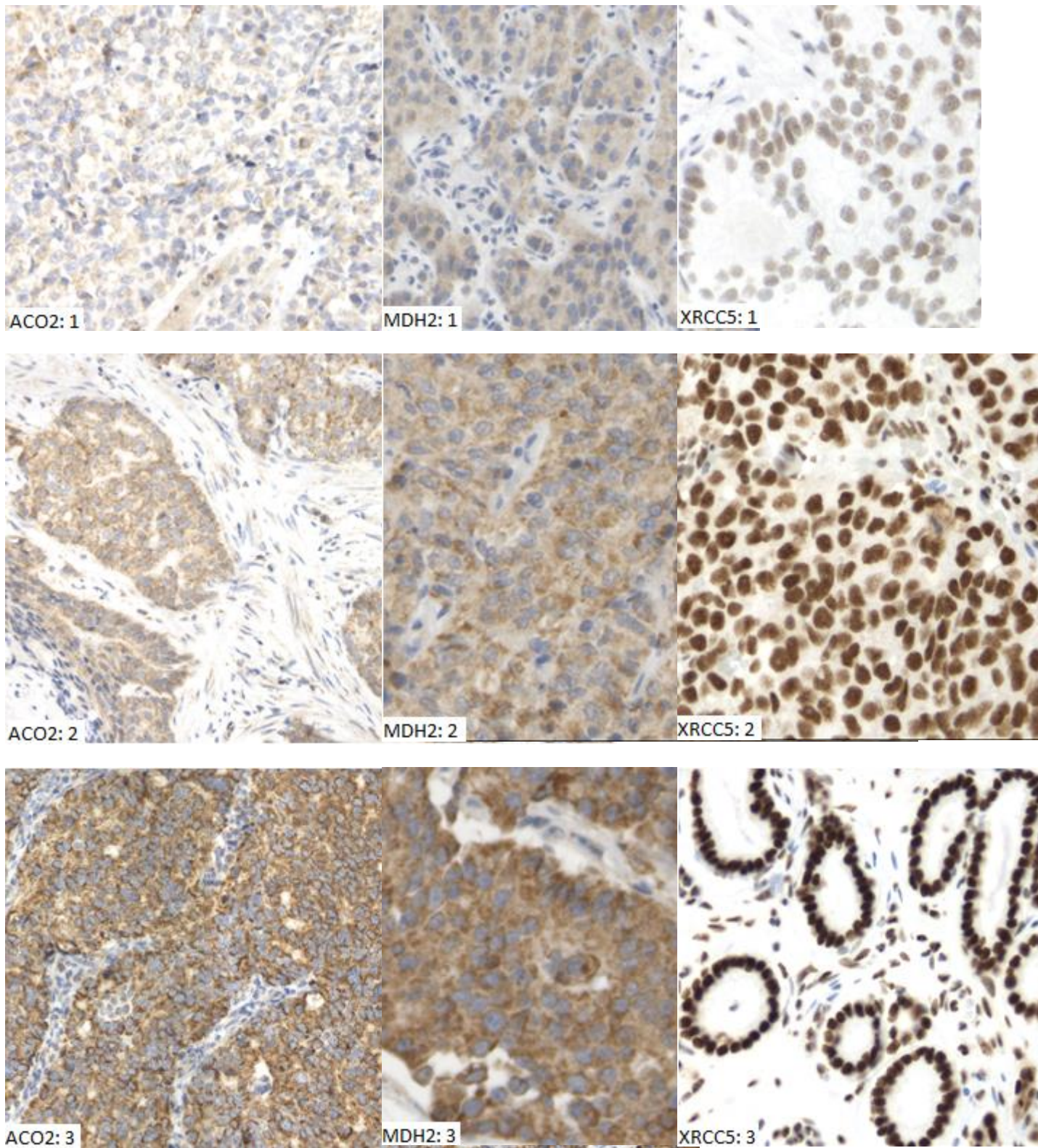


Figure 1. Examples of staining scores 1, 2 and 3 with ACO2, MDH2 and XRCC5 expression. First vertical column represents ACO2, second MDH2 and third XRCC5.

To keep the scoring consistent, the grading was done three times. Unclear, destroyed or non-cancerous specimens were excluded from the analyses.

2.3 Statistical analyses

The purpose of this study was to examine if aforementioned proteins (*ACO2*, *MDH2* and *XRCC5*) have different expression levels in prostate cancer and if these expression differences are connected to common diagnostic markers in prostate cancer. Data for these variables has been previously collected. Analyses were done using Microsoft Office Excel 2016- and GraphPad Prism 6-sofwarees.

Staining score was compared to PSA-score, Gleason-score, progression free months, survival until disease progression, age at diagnosis, tumor's T-stage evaluated by a pathologist and Ki-67 expression level. T-stage and Ki-67 was studied also in CRPC-specimens. Staining intensities between primary prostatectomy and CRPC-samples were also compared to see if statistically significant differences existed.

One-way ANOVA's Kruskal-Wallis test was used when comparing protein expression in primary prostatectomy and PSA, Gleason, progression free months, age at diagnosis and tumor's T-stage in CRPC. T-test's Mann Whitney test was used for tumor's T-stage and Ki-67 expression in primary prostatectomy samples and for Ki-67 expression in CRPC-specimens. Mann-Whitney was also used when comparing scores between primary prostatectomy and CRPC-samples.

Survival was also studied with Log-rank's Mantel-Cox test. The endpoint was defined as the moment of disease progression to hormone-refractory type.

3 RESULTS

The total number of samples stained was 1 665, of which 544 primary prostatectomy samples and 160 castration-resistant prostate cancer specimens were accepted to the subsequent analyses. More specific information about the distribution of the scores among the proteins and the individual number of samples are presented on table 1 for primary prostatectomy and on table 2 for CRPC-specimens.

Table 1. Primary prostatectomy scores.

Proteins	Expression score				Total	Excluded
	0	1	2	3		
XRCC5	0 (0 %)	30 (15 %)	105 (52 %)	66 (33 %)	201 (100 %)	125
MDH2	29 (18 %)	59 (39 %)	50 (33 %)	15 (10 %)	153 (100 %)	173
ACO2	13 (7 %)	49 (26 %)	92 (48 %)	36 (19 %)	190 (100 %)	136

From table 1 it can be seen that expression score 2 was most common in *XRCC5* and *ACO2* whereas in *MDH2* the score 1 was most prevalent. The portion of samples excluded was quite high which reduced the statistical strength and affected the significance of the results as we can see later.

Table 2. Castration-resistant prostate cancer scores.

Proteins	Expression score				Total	Excluded
	0	1	2	3		
XRCC5	0 (0 %)	4 (12 %)	18 (55 %)	11 (33 %)	33 (100 %)	191
MDH2	5 (8 %)	19 (30 %)	34 (54 %)	5 (8 %)	63 (100 %)	156
ACO2	7 (11 %)	19 (30 %)	31 (48 %)	7 (11 %)	64 (100 %)	161

The score 2 was clearly the most prevalent among all proteins in CRPC as visualised in table 2. The portion of samples excluded was even greater in CRPC than in prostatectomy samples. The differences in distribution between PP and CRPC scores were also studied. These results are presented as a graph in figure 2. No statistically significant relationship was found between scores given to PP or CRPC samples.

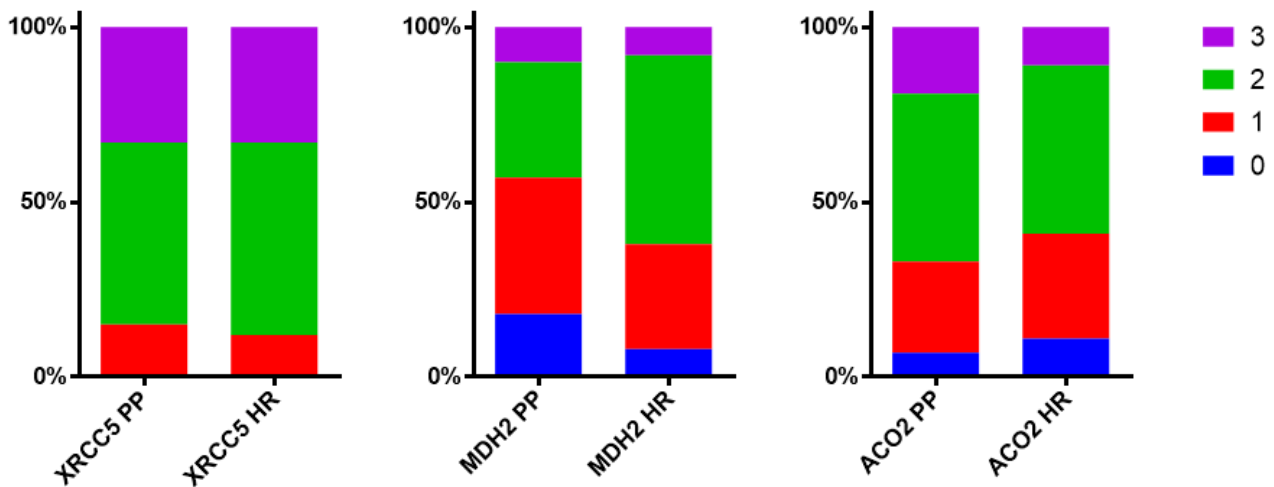


Figure 2. Stacked column graphs comparing expression scores given to primary prostatectomy (PP) and hormone-refractory (HR) samples.

The scores given were a bit higher for hormone-refractory (HR) samples with *XRCC5* and *MDH2* but not in *ACO2*. Nevertheless, the differences were too small to be of statistical significance.

Results comparing expression scores and common variables are presented on table 3 for *XRCC5*, on table 4 for *MDH2* and on table 5 for *ACO2* with means and standard deviations (SD) along with the P-values. Only Ki-67 expression in primary prostatectomy samples with *ACO2* and *MDH2* scores had a statistically significant result.

Table 3. Results from *XRCC5*

Variables	Expression score			P-value
	1	2	3	
PSA (ng/ml)	14.09 ± 11.16	14.88 ± 11.06	12.19 ± 9.107	0.2821
Progression free months	90.76 ± 65.51	95.52 ± 74.51	100.4 ± 68.12	0.7603
Age at diagnosis	65.06 ± 5.648	62.81 ± 4.850	63.74 ± 4.331	0.2045
Ki-67 CRPC	30.33 ± 10.26	13.13 ± 9.612	14.00 ± 8.106	0.0755
Ki-67 PP	11.59 ± 12.25	6.435 ± 5.213	7.259 ± 4.577	0.0941
T-stage PP	2: 23(79%), 3: 6(21%)	2: 105(67%), 3: 35(33%)	2: 46(70%), 3: 20(30%)	0.6015

There were no significant results found when studying *XRCC5* expression compared to common variables, although Ki-67 expression was almost statistically significant (P-value < 0.05). The pattern in Ki-67 expression was however divergent from the ones seen with *ACO2* and *MDH2* because the expression levels were higher on smaller protein expression scores. PSA's and progression free months' mean values increased with the expression scores but the differences were so small that they fit inside the standard deviations.

Table 4. MDH2 results

Variables	Expression score			P-value
	1	2	3	
PSA (ng/ml)	15.20 ± 12.68	19.51 ± 34.71	11.29 ± 4.433	0.9804
Progression free months	99.12 ± 70.50	90.44 ± 69.59	101.5 ± 62.92	0.6920
Age at diagnosis	62.62 ± 5.399	63.60 ± 4.967	62.12 ± 6.638	0.6258
Ki-67 CRPC	14.94 ± 9.010	16.97 ± 10.28		0.5747
Ki-67 PP	7.189 ± 6.677	12.17 ± 16.07		0.0362
T-stage PP	2: 42(71%), 3: 17(29%)	2: 31(62%), 3: 19(38%)	2: 10(67%), 3: 5(33%)	0.4306

With *MDH2* the Ki-67 PP expression was statistically significant. The scores 2 and 3 were combined in Ki-67 expression because the amount of values was then more equal between score groups. The expression score was higher in primary prostatectomies with T-stage 2 than T-stage 3 but the difference was too small to be of significance.

Table 5. The results from ACO2

Variables	Expression score			P-value
	1	2	3	
PSA (ng/ml)	16.19 ± 14.81	15.84 ± 26.14	13.38 ± 9.299	0.7950
Progression free months	79.60 ± 66.76	96.17 ± 71.54	92.07 ± 67.44	0.5086
Age at diagnosis	63.08 ± 5.080	62.68 ± 5.724	64.14 ± 3.983	0.7344
Ki-67 CRPC	19.12 ± 10.51	15.30 ± 9.968		0.2098
Ki-67 PP	6.238 ± 4.838	10.24 ± 12.89	9.438 ± 8.628	0.0506
T-stage PP	2: 36(73%), 3: 13(27%)	2: 63(68%), 3: 29(32%)	2: 23(64%), 3: 13(36%)	0.3407

Ki-67 PP was almost significant with P-value 0.0506 when the scores 2 and 3 were handled separately. When the scores 2 and 3 were combined as was done with *MDH2* previously, the P-value became 0.0147 and the result was statistically significant.

Gleason scores are presented on tables 6, 7 and 8 for *XRCC5*, *MDH2* and *ACO2*, respectively. This is because Gleason grade was handled as a categorical variable with score distribution studied. T-stage was also handled as a categorical variable but these results are presented on tables 3, 4 and 5 nevertheless.

Table 6. The Gleason scores for XRCC5 in primary prostatectomy samples.

Gleason	Expression score			Total	P-value
	1	2	3		
< 7	12 (14 %)	40 (49 %)	30 (37 %)	82 (100 %)	
7	15 (16 %)	52 (55 %)	27 (29 %)	94 (100 %)	
> 7	3 (13 %)	13 (57 %)	7 (30 %)	23 (100 %)	
					0.7519

Table 7. The Gleason scores for MDH2 in primary prostatectomy samples.

Gleason	Expression score			Total	P-value
	1	2	3		
< 7	20 (45 %)	17 (39 %)	7 (16 %)	44 (100 %)	
7	3 (43 %)	4 (57 %)	0 (0 %)	7 (100 %)	
> 7	17 (46 %)	13 (35 %)	7 (19 %)	37 (100 %)	
					0.7029

Table 8. The Gleason scores for ACO2 in primary prostatectomy samples.

Gleason	Expression score			Total	P-value
	1	2	3		
< 7	19 (28 %)	35 (51 %)	14 (21 %)	68 (100 %)	
7	24 (28 %)	47 (54 %)	16 (18 %)	87 (100 %)	
> 7	5 (25 %)	9 (45 %)	6 (30 %)	20 (100 %)	
					0.8935

There was no statistical difference between protein expression score and the Gleason grade given. The figures visualising the relationship between PP Ki-67 expression and ACO2 and MDH2 protein expression scores are presented in figure 2.

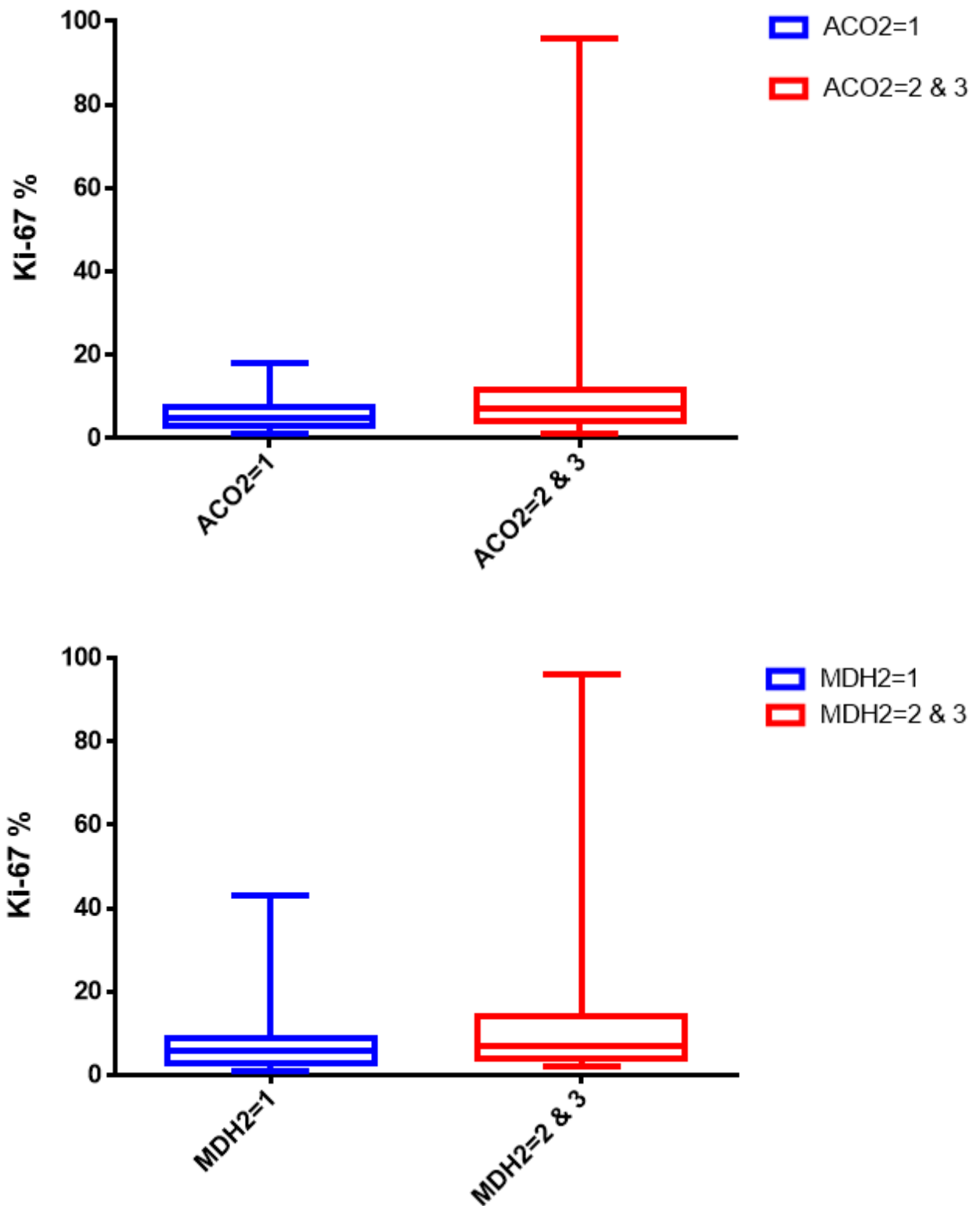


Figure 2. The relationship between Ki-67 expression and ACO2 and MDH2 scores in primary prostatectomy samples. Box represents the standard deviation with mean value as the line and the whiskers visualise maximum and minimum values.

Survival curves are presented on figure 3. Different expression levels are visualised in different colours.

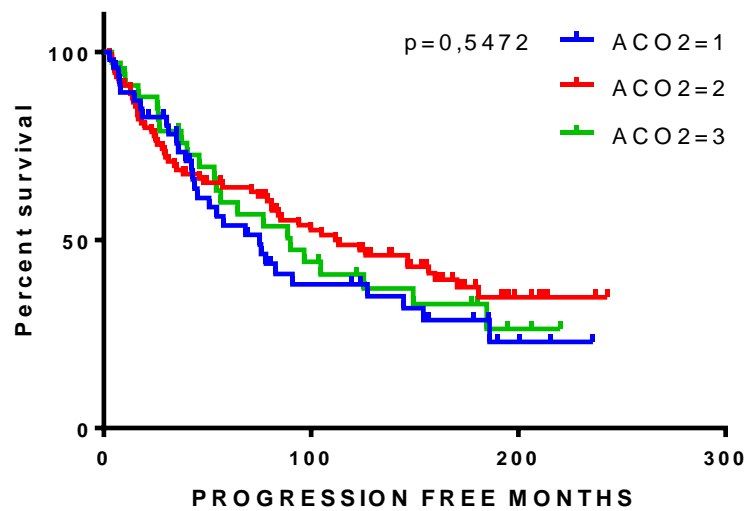
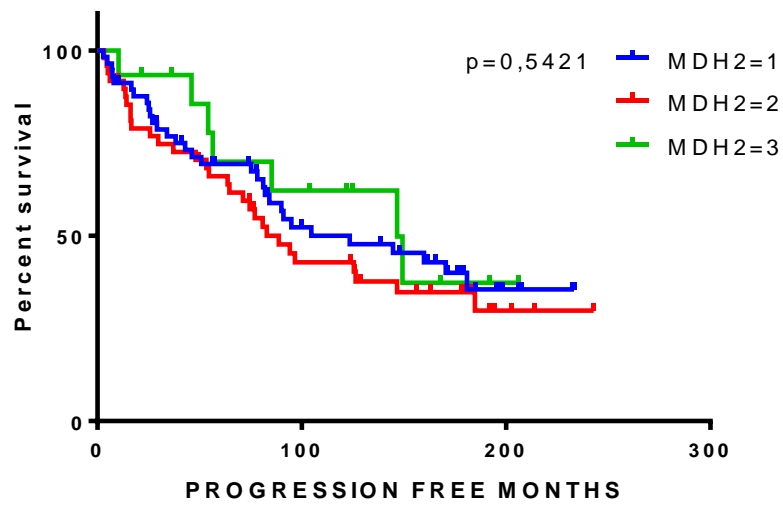
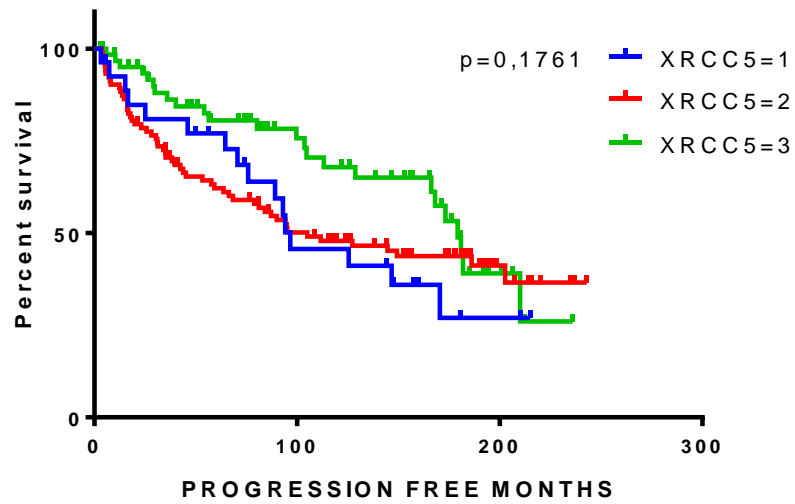


Figure 3. Survival curves comparing disease progression in primary prostatectomy samples.

The survival curves did not differ significantly and median survival did not correlate to different protein expression levels. The results are analysed more in the following discussion-section.

4 DISCUSSION

These analyses conducted found a possible association with *ACO2* and *MDH2* with prostate cancer according to the stronger Ki-67 expression with higher staining grades. There have been many studies validating aberrant mitochondrial functions in prostate cancer (13,15,19,20), although some have suggested that for example the abnormal aconitase functions are connected to the faulty zinc metabolism (16). Metabolic enzymes are controlled by many intracellular pathways. Enzymes can therefore be more active even if their levels aren't increased that noticeably (14).

Aconitase and malate dehydrogenase are connected to each other as both affect the rate of mitosis (21) and malate dehydrogenase is controlled allosterically by citrate, the substrate for aconitase (18). Malate dehydrogenase has been found to be over-expressed in many cancers and it has many functions beneficial to the cancer like improving energy status and removing cytostatics from the cells (19,20).

Ki-67 is a proliferation marker and it has a negligible expression in normal prostatic tissue. It is though active in cells going through cell cycle and hence very prominent in actively dividing cancer cells. (28). It has also been implicated as a good prognostic factor for the treatment decisions comparing radical or conservative therapy in prostate cancer (29). Ki-67 has also been implicated as a promising prognostic factor for the metastasis and cancer specific death in prostate cancer (30,31).

This study consisted of previously obtained TMA slides which were stained with different antibodies. Statistical significance would probably have been higher with more undamaged specimens because a large part of the slides contained destroyed or missing spots. Larger specimen sizes will be needed for the full confirmation of the role of *XRCC5*, *MDH2* and *ACO2* in prostate cancer. To verify the results, several people should make the scoring to decrease its subjectivity.

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